

Published in final edited form as:

Anal Chem. 2013 February 5; 85(3): 1540–1546. doi:10.1021/ac3037206.

Mechanistic evaluation of the pros and cons of digital RT-LAMP for HIV-1 viral load quantification on a microfluidic device and improved efficiency via a two-step digital protocol

Bing Sun[†], Feng Shen[†], Stephanie E. McCalla[†], Jason E. Kreutz[§], Mikhail A. Karymov[†], and Rustem F. Ismagilov^{†,*}

[†]Division of Chemistry and Chemical Engineering, California Institute of Technology, 1200 East California Boulevard, Pasadena, California 91125, United States

Abstract

Here we used a SlipChip microfluidic device to evaluate the performance of digital reverse transcription-loop mediated isothermal amplification (dRT-LAMP) for quantification of HIV viral RNA. Tests are needed for monitoring HIV viral load to control the emergence of drug resistance and to diagnose acute HIV infections. In resource-limited settings, *in vitro* measurement of HIV viral load in a simple format is especially needed, and single-molecule counting using a digital format could provide a potential solution. We showed here that when one-step dRT-LAMP is used for quantification of HIV RNA, the digital count is lower than expected and is limited by the yield of desired cDNA. We were able to overcome the limitations by developing a microfluidic protocol to manipulate many single molecules in parallel through a two-step digital process. In the first step we compartmentalize the individual RNA molecules (based on Poisson statistics) and perform reverse transcription on each RNA molecule independently to produce DNA. In the second step, we perform the LAMP amplification on all individual DNA molecules in parallel. Using this new protocol, we increased the absolute efficiency (the ratio between the concentration calculated from the actual count and the expected concentration) of dRT-LAMP 10-fold, from ~2 % to ~23 %, by i) using a more efficient reverse transcriptase, ii) introducing RNase H to break up the DNA:RNA hybrid, and iii) adding only the BIP primer during the RT step. We also used this two-step method to quantify HIV RNA purified from 4 patient samples and found that in some cases, the quantification results were highly sensitive to the sequence of the patient's HIV RNA. We learned the following three lessons from this work: i) digital amplification technologies, including dLAMP and dPCR, may give adequate dilution curves and yet have low efficiency, thereby providing quantification values that underestimate the true concentration. Careful validation is essential before a method is considered to provide absolute quantification; ii) the sensitivity of dLAMP to the sequence of the target nucleic acid necessitates additional validation with patient samples carrying the full spectrum of mutations; and iii) for multistep digital amplification chemistries, such as a combination of reverse transcription with amplification, microfluidic devices may be used to decouple these steps from one another and to perform them under different, individually optimized conditions for improved efficiency.

*Corresponding Author: rustem.admin@caltech.edu.

[†]Present Address SlipChip, LLC, 129 N Hill Ave, Pasadena, CA, 91106, United States

[§]Present Address Department of Chemistry, University of Washington, Seattle, Washington 98195, United States

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions B.S., F.S., S.E.M., and R.F.I. designed the SlipChip experiments. B.S. performed experiments and data analysis. S.E.M. did ssDNA synthesis. B.S., S.E.M., and R.F.I. wrote the paper. J.E.K. contributed to the design of the two-step SlipChip and data analysis. M.A.K. provided suggestions on experiment design.

Disclosure: F.S. and R.F.I. have a financial interest in SlipChip LLC.

Introduction

In this paper, we evaluate the potential of using digital reverse transcription loop-mediated isothermal amplification (dRT-LAMP) for quantifying the concentration of HIV-1 viral RNA. A simple, convenient quantification method for HIV viral load is important as an *in vitro* diagnostic tool to aid the 34 million people worldwide with HIV, many of whom live in developing countries and under resource-limited conditions.¹ As antiretroviral treatment becomes more widely available, viral resistance to first-line antiretroviral drugs is a problem of increasing concern. To combat the spread of drug resistance and make appropriate choices concerning second-line therapy, it is important for patients to undergo regular monitoring of HIV viral load (every 3–4 months)² to check for sharp increases, which indicate the evolution of viral resistance to first-line therapies. Real-time reverse transcription-polymerase chain reaction (RT-PCR) is the most commonly adopted method in reference laboratories, yet it faces multiple challenges in resource-limited conditions because of the cost and technical constraints. It requires dedicated equipment and physical resources such as uninterrupted electricity, air conditioning, refrigeration, an isolated room, and access to clean water.³ Moreover, it is a kinetic measurement method and is sensitive to variations in reaction conditions that influence reaction rate. As a result, the reproducibility of this method can be influenced by a variety of perturbations (fluctuations in sample quality, reaction time, and temperature), which are potential concerns for HIV viral load quantification, especially in settings where little control over environmental fluctuations is available.⁴

Here we ask whether dRT-LAMP quantification provides an attractive alternative to real-time RT-PCR. In principle, a digital format quantification method could have higher tolerance to variations in reaction rate than real-time bulk measurements because it provides quantitative information via many qualitative yes/no experiments. As is shown in the case of recombinase polymerase amplification (RPA),⁵ a digital format quantification method is robust to temperature variation, because it requires interpreting only endpoint yes/no results. Moreover, the LAMP reaction can be performed under isothermal conditions and, when calcein is used as an indicator to produce bright fluorescent output, it could allow for easy counting of digital output, either visually or with a portable imaging device such as a cell phone camera instead of a sophisticated fluorescence microscope. This unique property makes this chemistry a viable candidate for resource-limited settings.

In this paper, we used a microfluidic SlipChip⁶ to evaluate the suitability of dRT-LAMP for quantifying HIV viral RNA. It has previously been shown that multiplexed PCR,⁷ dPCR,⁸ dRT-PCR,⁹ and dRPA⁵ can be performed successfully on SlipChip. In addition, digital LAMP (but not digital RT-LAMP) has previously been demonstrated for model double-stranded DNA (dsDNA) targets on other microfluidic devices.^{10,11} However, we found here that performing dRT-LAMP in a single step on SlipChip is problematic for quantifying HIV viral RNA: the quantification result using dRT-LAMP was only 2.0 % of that achieved using dRT-PCR with the same template concentration. While bulk RT-LAMP assays for the detection of HIV-1 RNA have been developed,^{12,13} these assays differ from digital assays because the latter is performed at the single-molecule level, which might introduce additional complexity to the reaction. We performed mechanistic studies to understand some of the shortcomings of one-step dRT-LAMP and developed a two-step dRT-LAMP protocol on SlipChip to overcome these limitations. We demonstrated that SlipChip can be used to compartmentalize single molecules and then to implement multi-step reactions on those compartments. We further showed that dRT-LAMP can be performed on SlipChip devices made of both glass and plastic, which is a more desirable and cost-effective material for resource-limited settings. Finally, we identified problems—including low overall efficiency

and sensitivity to mutations in patient samples—that need to be addressed in the future to make this method suitable for quantitative diagnostics.

Experimental Section

Chemicals and materials used in this paper are described in the SI. The fabrication, assembly, and loading protocols for glass and plastic SlipChip are based on previous work^{6,9} and are described in the SI. HIV viral RNA purification from AcroMetrix® HIV-1 Panel Copies/ml was done using the iPrep™ purification instrument. See SI for more information. Primer sequences for RT-LAMP and RT-PCR amplification and the sequencing results of HIV viral RNA used in the paper are described in the SI.

ssDNA synthesis

HIV cDNA was created by reverse transcription of the purified AcroMetrix® HIV RNA using the SuperScript III First-Strand Synthesis SuperMix according to the manufacturer's instructions. Briefly, a mixture of purified HIV RNA (10-fold diluted from the direct elution), 100 nM B3 primer, 1× Annealing buffer, and water were heated to 65 °C for 5 minutes and then placed on ice for 1 minute. A reaction mix and SuperScript III/RNase Out enzyme mix were added to the reaction for a final volume of 40 µL, and the mixture was placed at 50 °C for 50 minutes. The mixture was then heated to 85 °C for 5 minutes to deactivate the reverse transcriptase, chilled on ice, split into 5 µL aliquots, and frozen at −20 °C until further use. Biotin-labeled DNA was created in a PCR reaction containing a 1:50 dilution of the HIV cDNA, 500 nM biotin-B3 and F3 primers, 500 µM dNTPs, 1 U/µL Phusion DNA polymerase and 1× of the associated HF buffer mix. After an initial 1 minute enzyme activation step at 98 °C, the reaction was cycled 39 times at 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s, and finished with a 5 minute polishing step at 72 °C. The resulting DNA product was run on a 1.2% agarose gel in TBE buffer stained with 0.5 µg/mL ethidium bromide. The specific band was cut out and purified using the Wizard SV gel and PCR cleanup kit according to the manufacturer's instructions and eluted into 50 µL of nuclease-free water. 50 µL of streptavidin MyOne T1 magnetic beads were primed by slow-tilt rotation for 24 hours in 20 mM NaOH¹⁴. The beads were washed 1 time with water and 4 times with binding buffer (5 mM Tris, 0.5 mM EDTA, 1 M NaCl, 0.05% Tween-20) and re-suspended in 30 µL of 2× concentrated binding buffer. 30 µL of PCR product was added to the beads and incubated for 15 minutes while gently rotating to allow binding of the DNA to the magnetic beads. The beads were separated with a magnet, the supernatant was removed, and the beads were re-suspended in 40 µL of 20 mM NaOH and incubated for 10 minutes on a rotator to separate the non-biotinylated strand. The beads were then separated with a magnet, and the supernatant containing the ssDNA was collected and mixed with 20 µL of 40 mM HCl. The resulting ssDNA was then purified using an ssDNA/RNA cleaner and concentrator kit, eluted in 20 µL water, and run on an Agilent RNA nano bioanalyzer to confirm the size and integrity of the final product.

dRT-LAMP and dLAMP amplification on SlipChip

To amplify HIV viral RNA using the one-step RT-LAMP method, the RT-LAMP mix contained the following: 20 µL RM, 2 µL BSA (20 mg/mL), 2 µL EM, 2 µL FD, 2 µL of primer mixture (20 µM BIP/FIP, 10 µM LooP_B/Loop_F and 2.5 µM B3/F3), various amount of template solution, and enough nuclease-free water bring the volume to 40 µL. The solution was loaded onto a SlipChip and heated at 63 °C for 60 minutes.

To amplify HIV viral RNA using the two-step RT-LAMP method, the first solution contained the following: 10 µL RM, 1 µL BSA, 0.5 µL EXPRESS SYBR® GreenER™ RT module (part of EXPRESS One-Step SYBR® GreenER™ Universal), 0.5 µL BIP primer

(10 μ M), various amount of, and enough nuclease-free water to bring the volume to 20 μ L. The second solution contained: 10 μ L RM, 1 μ L BSA, 2 μ L DNA polymerase solution (from LoopAmp® DNA amplification kit), 1 μ L or 2 μ L FD, 2 μ L other primer mixture (20 μ M FIP, 17.5 μ M FIP, 10 μ M LooP_B/Loop_F and 2.5 μ M F3), 1 μ L Hybridase™ Thermostable RNase H, and enough nuclease-free water to bring the volume to 20 μ L. The first solution was loaded onto a SlipChip device and incubated at 37 °C or 50 °C, then the second solution was loaded onto the same device and mixed with the first solution, and the entire device was incubated at 63 °C for 60 minutes.

To amplify λ -DNA, the LAMP mix contained the following: 20 μ L RM, 2 μ L BSA (20 mg/mL), 2 μ L DNA polymerase, 2 μ L FD, 2 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M LooP_B/Loop_F and 2.5 μ M B3/F3), various amount of template solution, and enough nuclease-free water to bring the volume to 40 μ L. The same loading protocol as above was performed and the device was incubated at 63 °C for 70 minutes.

To amplify ssDNA, the LAMP mix contained the following: 20 μ L RM, 2 μ L BSA, 2 μ L DNA polymerase, 2 μ L FD, 2 μ L of primer mixture (20 μ M BIP/FIP, 10 μ M LooP_B/Loop_F and 2.5 μ M B3/F3), various amount of template solution, and enough nuclease-free water to bring the volume to 40 μ L. The same loading protocol as above was performed and the device was incubated at 63 °C for 60 minutes.

dRT-PCR amplification on SlipChip

To amplify HIV viral RNA, the RT PCR mix contained the following: 20 μ L 2X Evagreen SuperMix, 2 μ L BSA, 1 μ L EXPRESS SYBR® GreenER™ RT module, 1 μ L each primer (10 μ M), 2 μ L template, and enough nuclease-free water to bring the volume to 40 μ L. The amplification was performed at the same conditions as reported before⁹ except for a shortened reverse transcription step of 10 minutes.

Image acquisition and analysis methods are described in the SI.

Results and discussion

We first compared quantification results of HIV viral RNA at four dilutions using two different digital chemistries—dRT-PCR and dRT-LAMP. dRT-PCR with primers targeting the LTR region of HIV was used as the standard because it has been validated previously.⁹ B3/F3 primers taken from the LAMP primer set were also used in dRT-PCR for comparison. For RT-LAMP, we used a commercial kit with published primers targeting the p24 gene.¹² The priming region of HIV RNA was sequenced to confirm that there was no mismatch between primers and template (see SI). HIV viral RNA concentration was calculated based on the number of observed positive wells (“digital counts”) on a single device according to the Poisson statistical analysis method discussed in a previous paper.¹⁵ The overall result was surprising: while dilution curves with good linearity were obtained for both digital chemistries listed above, the absolute values were dramatically different. For the two chemistries, the same concentration of RNA gave quantification values that differed by ~50 fold, with dRT-LAMP giving the lowest digital counts (Figure 1). The quantification values calculated from digital counts for one-step dRT-LAMP were on average only 2.0% of the values for the dRT-PCR standard with LTR primers and were on average 3.9% of the values for dRT-PCR with B3/F3 primers. One could interpret these results optimistically and state that as long as one has a calibration curve, one could perform adequate quantification no matter the efficiency of the particular digital amplification chemistry. However, there are also two alternative interpretations, admittedly more pessimistic in nature. The first: seeing a proper dilution curve does not mean that the quantification is correct—digital “absolute” quantification is not absolute unless rigorously validated by independent experiments.

Second, using an inefficient digital method like the one-step dRT-LAMP shown in Figure 1 is inadequate for quantifying lower concentrations of analytes. This is a problem, especially in the case of HIV viral load, where changes most often in the range of 50 to 5,000 copies of RNA per mL need to be quantified to make clinical decisions.¹⁶ Therefore, we acted on the more pessimistic interpretation of these results and worked on understanding why the digital efficiency of dRT-LAMP is so low, towards the goal of improving its efficiency.

Given the poor performance of one-step dRT-LAMP, we performed a parallel control experiment on the same SlipChip using a published and well-characterized LAMP system based on λ -DNA^{10,17} to confirm that the SlipChip device did not introduce any artifacts when used with LAMP chemistry. The concentration of the λ -DNA stock solution was measured with a Qubit fluorometer and used as the expected original concentration for further dilution. dLAMP was performed according to a previous publication.¹⁰ Without a pre-heating step at 95 °C to dissociate double-stranded DNA template, we observed zero digital counts. Unfortunately, previous reports on dLAMP with λ -DNA did not specify whether pre-heating was or was not required.^{10,16} Neither was this pre-heating step specified in a recent paper where β -actin DNA was used as the template for dLAMP reaction.¹¹ When a pre-heating step was introduced, the quantification value of dLAMP on SlipChip increased to 62% of the expected value (Figure S2). The results depend on the details of how pre-heating is performed and how quickly DNA is cooled and used in the subsequent amplification step.^{18,19} We did not optimize the pre-heating protocol since it is not required by single-stranded HIV RNA (aside from the secondary structure), so we did not expect a perfectly efficient quantification. It was concluded that the glass SlipChip platform was not introducing artifacts significant enough to be responsible for the 2.0% efficiency observed for one-step dRT-LAMP.

To determine the cause of the lower efficiency of one-step dRT-LAMP compared to dRT-PCR, we attempted to decouple the reverse transcription step from the amplification step and look at the efficiency of each step independently. We designed a glass SlipChip device for performing dRT-LAMP in two steps (Figure 2). The device was composed of two glass plates with wells and channels etched on their facing sides. The plates of the chip were assembled and aligned to allow for the loading, compartmentalization, incubation, and mixing of reagents in multiple steps. This chip is reminiscent of, but not the same as, the chip previously described for digital RPA.⁵ First, a buffered solution containing template, primer, and RT enzyme was loaded into wells on the chip (Figure 2B). Next, the plates of the chip were slipped relative to one another to confine single HIV viral RNA molecules into droplets (Figure 2C). A first incubation step was performed here to allow for reverse transcription. cDNA was synthesized from RNA in each compartment during the reverse transcription step. Then, a second solution containing the LAMP reagent mixture and other primers was loaded (Figure 2D) and split into compartments by slipping (Figure 2E). Finally, each of the compartments containing a cDNA molecule was combined with a compartment containing LAMP reagents, and the entire device was incubated at a higher temperature for amplification. The fluorescence microphotograph of part of a loaded glass device after amplification, along with a line scan showing intensity, is presented in the SI.

We then studied the influence of several factors, including the orders of primers annealing to the RNA template, the effect of different reverse transcriptases, and the ease of breaking up the DNA:RNA hybrid independently, and proposed a new two-step mechanism (Figure 3).

In the RT-LAMP mechanism, a BIP primer should first hybridize to target RNA to start the synthesis of the first-strand cDNA, which will be released from the RNA later by strand displacement initiated by a B3 primer.^{20,21} However, we hypothesized that if instead of BIP primer, Loop_B primer or B3 primer hybridizes to the target RNA first and starts reverse

transcription, no looped structure on the 3' size of cDNA will be formed after strand displacement; and once B3 primer hybridizes to RNA and forms an RNA:DNA duplex, it is difficult for other primers to insert into the duplex and start reverse transcription because of the high stability of RNA:DNA.²² As a result, we hypothesized that it is critical to start reverse transcription with BIP primer to make sure each RNA molecule is amplified. We tested this hypothesis by performing a two-step protocol and using different primer compositions in the RT step: one has all LAMP primers and the other has only BIP primer (Figure 3A). With all primers, 3.2 % efficiency relative to dRT-PCR using LTR primers was observed, while with only BIP primer, the efficiency of dRT-LAMP increased about 2 fold to 6.8 % (Figure 3B ii and iii). All of the dRT-LAMP experiments reported in Figure 3B were performed using an HIV RNA solution of the same concentration as the 0.1 dilution in Figure 1B. Given these results, we concluded that the order in which the primers anneal to the target influences the initiation efficiency of the reaction.

Different reverse transcriptases are reported to have different efficiencies in first-strand cDNA synthesis.²³ In bulk experiments where multiple copies of the target are initially present, a high RT efficiency is not crucial for the reaction to proceed because the LAMP reaction is so sensitive that even with a very low RT yield, the synthesized cDNA molecules can still initiate subsequent amplification. This could also explain why RT-LAMP can be performed at a temperature (63 °C) beyond the optimal temperature of AMV reverse transcriptase (37 °C – 42 °C). However, at the single-molecule level, to get an accurate absolute digital count of RNA molecules, it is necessary to choose a reverse transcriptase with a high RT yield to obtain cDNA from as many RNA molecules as possible (and ideally, from each molecule) in the reverse transcription step. We replaced AMV reverse transcriptase with Superscript III reverse transcriptase, which is reported to have a higher reverse transcription efficiency.²³ However, we did not observe any efficiency increase (Figure 3B iii and iv). Considering the fact that Superscript III (similar to Superscript II) has low strand displacement activity²⁴ due to the reduced RNase H activity, while AMV reverse transcriptase has strong strand displacement activity, and that the release of cDNA from the RNA:cDNA hybrid relies on the strand displacement initiated by B3 primer, we hypothesized that even if more cDNA is synthesized by Superscript III, it may not be released from the hybrid. To test this hypothesis, we added RNase H, which can cleave RNA from an RNA:DNA duplex through a hydrolytic mechanism, to aid the release of the cDNA (Figure 3A). However, because the presence of RNase H will interfere with the activity of reverse transcriptase, it can only be added after the reverse transcription is complete. We used the two-step protocol and added a thermally stable RNase H to the second reaction mixture. To make sure we are looking at only the effect of RNase H breaking up the RNA:DNA hybrid, without the interference from any strand displacement, we removed the B3 primer from the second reaction mixture since it was no longer needed. Both reverse transcriptases were tested with RNase H in this protocol. We observed no improvement using RNase H together with AMV reverse transcriptase (Figure 4A v), confirming that AMV strand displacement activity was sufficient for the release of the first strand ssDNA. However, under the same conditions, using RNase H together with Superscript III significantly increased the quantification results of dRT-LAMP from 5.3 % to 25 % (Figure 3B vi) relative to that quantified by dRT-PCR with LTR primers.

In a LAMP reaction, calcein is used as the readout fluorophore.²⁵ Yet because calcein is a good ligand for metal ions, it might interfere with enzyme activities or dsDNA stability. We tested the effect of adding different amounts of calcein and found that decreasing the calcein to half (0.5X) of the recommended concentration gave the highest efficiency (Figure 3B vii). We also performed two-step dRT-LAMP in the temperature range of 57 °C to 66 °C and determined the optimal temperature to be 63 °C (data not shown).

The assay was reproducible in our hand and we believe that $n=2$ in each reaction condition is sufficient for purposes of optimizing the reaction protocols. However, to confirm the robustness of this protocol, we performed two-step dRT-LAMP with the optimized protocol at four different concentrations in quadruplicate and compared the results to one-step dRT-LAMP (Figure 3C). The quantification results showed a linear relationship with the dilution level for both protocols. The P value of the linear regression for one-step dRT-LAMP is 4.0×10^{-11} and for two-step dRT-LAMP is 7.4×10^{-9} , both of which are $\ll 0.01$. Therefore, we rejected the null hypothesis that there is no relationship between the data points that composed each curve. The average efficiency compared to dRT-PCR with LTR primers was $2.0\% \pm 0.2\%$ for one-step dRT-LAMP, and $23.2\% \pm 2\%$ for two-step dRT-LAMP; thus, it increased by about 10 fold.

Next, we excluded the influence of the RT step and assessed the efficiency of the LAMP step independently by performing dLAMP using a 225 nucleotide (nt) ssDNA of the same sequence as the p24 LAMP priming region as template. We hypothesized that if the formation of desired single-stranded cDNA is no longer the bottleneck in the two-step protocol, then dLAMP using ssDNA as template would have similar efficiency to two-step dRT-LAMP using HIV viral RNA as template. Because the 225 nt ssDNA did not include the LTR region, we could not use dRT-PCR with LTR primers as a reference with which to compare. Instead, we used dRT-PCR with B3/F3 primers as a reference. We have shown that two-step dRT-LAMP gave on average 39 % efficiency compared with dRT-PCR using B3/F3 primers in the serial dilution experiments (Figure 1). Here, we observed dLAMP using synthesized ssDNA as template gave 33 % efficiency compared to dPCR with B3/F3 primers using ssDNA as template. We assumed that dRT-PCR and dPCR should have the same efficiency because the HIV RNA template and the ssDNA template had the same sequence (see SI) and the same pair of primers was used to amplify them. Then, we concluded that two-step dRT-LAMP and dLAMP had comparable efficiency. This result indicated that using the two-step protocol, we have solved the problem of forming the desired single-stranded cDNA.

To evaluate the robustness of this dRT-LAMP chemistry in resource-limited settings, we also tested its compatibility with plastic material and its tolerance to sequence divergence of patient samples. We performed two-step dRT-LAMP of HIV viral RNA on a plastic SlipChip device with the same design as the glass device used in the previous experiments. The RNA quantification results using two-step dRT-LAMP on the plastic SlipChip device and the glass SlipChip device agreed well with each other (Figure 4A). This result indicated that the quantification results are not sensitive to the details of surface chemistry of the SlipChip, as we expected, because the surface chemistry control occurs at the liquid liquid interface of the aqueous solution and the lubricating fluid.^{6,7,26} This result also confirmed the possibility of using a potentially low-cost plastic device as the platform for performing dRT-LAMP.

We also tested the performance of two-step dRT-LAMP using HIV viral RNA purified from patient samples and compared these results to measurements from dRT-PCR. Plasma samples from four different patients were purified using a Roche TNAI kit. Both two-step dRT-LAMP with p24 primers and dRT-PCR with LTR primers were performed to quantify the RNA concentration. The dRT-LAMP quantification results were $43\% \pm 9\%$, $139\% \pm 33\%$, $37\% \pm 20\%$, and $0.9\% \pm 0.2\%$ relative to the corresponding dRT-PCR results, respectively (Figure 4B). The p24 region of the purified HIV viral RNA was sequenced (sequences listed in the SI). There were 3, 2, 5, and 5 point mutations in the priming regions of samples #1, 2, 3, and 4, respectively. Therefore, the general conclusion is that the efficiency of dRT-LAMP is sensitive to mutations in the target sequence, with more mutations leading to lower efficiency. In particular, for sample #4, according to the

sequencing results, two of the mutations are at the 3' end of the BIP priming region and are adjacent to each other, which we hypothesized dramatically decreased the efficiency of reaction. To test this hypothesis, we performed two-step dRT-LAMP on sample #4 with BIP primer corrected at these two positions and its efficiency increased from $0.9\% \pm 0.2\%$ to $22\% \pm 1\%$. Since a LAMP reaction requires three pairs of primers to proceed, its specificity is higher than that of PCR, which requires only one pair of primers. However, we speculate that this high specificity may also make the dLAMP method more sensitive to mutations in the priming regions. It would be valuable to take advantage of the high specificity of this method while maintaining good tolerance to point mutations in real patient samples. To achieve this goal, more than one set of primers could be included in a single assay to ensure a higher probability of accurate readings, although this idea needs to be tested experimentally.

Conclusion

We demonstrated in this paper that one-step dRT-LAMP for HIV viral RNA showed only 2.0% efficiency relative to that observed using dRT-PCR. In particular, we observed that the efficiency of dRT-LAMP for HIV viral RNA quantification is limited by the efficiency of reverse transcription, the sequence of primer annealing, and the efficiency of the breakup of DNA:RNA duplexes. We solved these problems by using a two-step approach that enabled us to use a more efficient reverse transcriptase, to add only the BIP primer during the RT step, and to introduce RNase H to break up the DNA:RNA hybrid. Technologies that enable multistep manipulation of many volumes in parallel, such as SlipChip, provide a platform for this multi-step protocol for dRT-LAMP. Such microfluidic technologies are useful for mechanistic studies of dLAMP and other digital, single-molecule reactions. Furthermore, the technologies that can be made simple enough for use in resource-limited settings would be useful for deploying digital single-molecule amplification for diagnostics applications.

Single-molecule counting using dLAMP and dRT-LAMP is attractive because it is isothermal and therefore does not require thermocycling equipment, is compatible with plastics, and provides a bright signal from the calcein detection system that should be readable by a cell phone. Although the efficiency of dRT-LAMP for quantification of HIV RNA has been improved over 10 fold, it ideally should be further improved by about 4 fold. A more significant problem is sensitivity of digital amplification to mutations in the target regions. This problem should be especially severe for viruses like HIV that mutate rapidly, and for other targets, the severity of this problem remains to be established. General solutions to this problem for dLAMP and dRT-LAMP protocols are needed. Furthermore, comparisons of the sensitivity of different digital amplification methods and their sensitivity to mutations found in a broad cohort of patient samples are also needed. Digital amplification technologies should not be assumed to provide absolute quantification until validated against an absolute standard and, for diagnostic applications, until validated on a representative set of patient samples. Should all of these issues be resolved, we are optimistic that digital single molecule technologies would become useful for quantifying molecules under resource-limited settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grant R01EB012946 administered by the National Institute of Biomedical Imaging and Bioengineering and by the NIH Director's Pioneer Award program, part of the NIH Roadmap for

Medical Research (5DP1OD003584). We thank Bridget Samuels for contributions to writing and editing this manuscript. We thank Dr. Loren Joseph and Dr. Reddy Poluru for providing RNA purified from patient samples.

References

- (1). WHO. UNAIDS. UNICEF. Global HIV/AIDS response: epidemic update and health sector progress towards universal access: progress report 2011. World Health Organization. 2011. http://www.who.int/hiv/pub/progress_report2011/summary_en.pdf
- (2). Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. 2012. <http://www.aidsinfo.nih.gov/contentfiles/lvguidelines/adultandadolescentgl.pdf>
- (3). Fiscus SA, Cheng B, Crowe SM, Demeter L, Jennings C, Miller V, Respass R, Stevens W, the Forum for Collaborative HIV Research Alternative Viral Load Assay Working Group. PLoS Med. 2006; 3:e417. [PubMed: 17032062]
- (4). Murphy J, Bustin SA. Expert Review of Molecular Diagnostics. 2009; 9:187. [PubMed: 19298142]
- (5). Shen F, Davydova EK, Du W, Kreutz JE, Piepenburg O, Ismagilov RF. Anal. Chem. 2011; 83:3533. [PubMed: 21476587]
- (6). Du W, Li L, Nichols KP, Ismagilov RF. Lab Chip. 2009; 9:2286. [PubMed: 19636458]
- (7). Shen F, Du W, Davydova EK, Karymov MA, Pandey J, Ismagilov RF. Anal. Chem. 2010; 82:4606. [PubMed: 20446698]
- (8). Shen F, Du W, Kreutz JE, Fok A, Ismagilov RF. Lab Chip. 2010; 10:2666. [PubMed: 20596567]
- (9). Shen F, Sun B, Kreutz JE, Davydova EK, Du W, Reddy PL, Joseph LJ, Ismagilov RF. J. Am. Chem. Soc. 2011; 133:17705. [PubMed: 21995644]
- (10). Gansen A, Herrick AM, Dimov IK, Lee LP, Chiu DT. Lab Chip. 2012; 12:2247. [PubMed: 22399016]
- (11). Zhu Q, Mu Y. Lab Chip. 2012; 12:4755. [PubMed: 22986619]
- (12). Curtis KA, Rudolph DL, Owen SM. J. Virol. Methods. 2008; 151:264. [PubMed: 18524393]
- (13). Hosaka N, Ndambi N, Ishizaki A, Kageyama S, Numazaki K, Ichimura H. J. Virol. Methods. 2009; 157:195. [PubMed: 19186193]
- (14). Wilson R. Nucleic Acid Ther. 2011; 21:437. [PubMed: 22047177]
- (15). Kreutz JE, Munson T, Huynh T, Shen F, Du W, Ismagilov RF. Anal. Chem. 2011; 83:8158. [PubMed: 21981344]
- (16). Calmy A, Ford N, Hirschel B, Reynolds SJ, Lynen L, Goemaere E, de la Vega FG, Perrin L, Rodriguez W. Clin. Infect. Dis. 2007; 44:128. [PubMed: 17143828]
- (17). Goto M, Honda E, Ogura A, Nomoto A, Hanaki KI. BioTechniques. 2009; 46:6.
- (18). Suzuki R, Ihira M, Enomoto Y, Yano H, Maruyama F, Emi N, Asano Y, Yoshikawa T. Microbiol. Immunol. 2010; 54:466. [PubMed: 20646211]
- (19). Geojith G, Dhanasekaran S, Chandran SP, Kenneth J. J. Microbiol. Methods. 2011; 84:71. [PubMed: 21047534]
- (20). Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Nucleic Acids Res. 2000; 28:e63. [PubMed: 10871386]
- (21). Nagamine K, Hase T, Notomi T. Mol. Cell. Probes. 2002; 16:223. [PubMed: 12144774]
- (22). Barone F, Cellai L, Matzeu M, Mazzei F, Pedone F. Biophys. Chem. 2000; 86:37. [PubMed: 11011698]
- (23). Ståhlberg A, Kubista M, Pfaffl M. Clin. Chem. 2004; 50:1678. [PubMed: 15331507]
- (24). Kelleher CD, Champoux JJ. J. Biol. Chem. 1998; 273:9976. [PubMed: 9545343]
- (25). Tomita N, Mori Y, Kanda H, Notomi T. Nat. Protocols. 2008; 3:877.
- (26). Li L, Du W, Ismagilov R. J. Am. Chem. Soc. 2009; 132:106. [PubMed: 20000708]

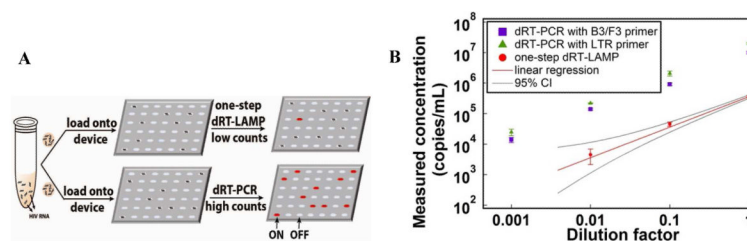


Figure 1.

One-step RT-LAMP has lower efficiency than RT-PCR in digital format when quantifying HIV RNA on SlipChip. (A) Schematic demonstration of one-step dRT-LAMP and dRT-PCR in which use of the same template concentration results in different digital counts. (B) Quantification results of HIV viral RNA (copies/mL) in a serial dilution experiment on SlipChip using one-step dRT-LAMP (n=4), dRT-PCR with B3/F3 primers (n=3), and dRT-PCR with LTR primers (n=3). Error bars represent standard deviation.

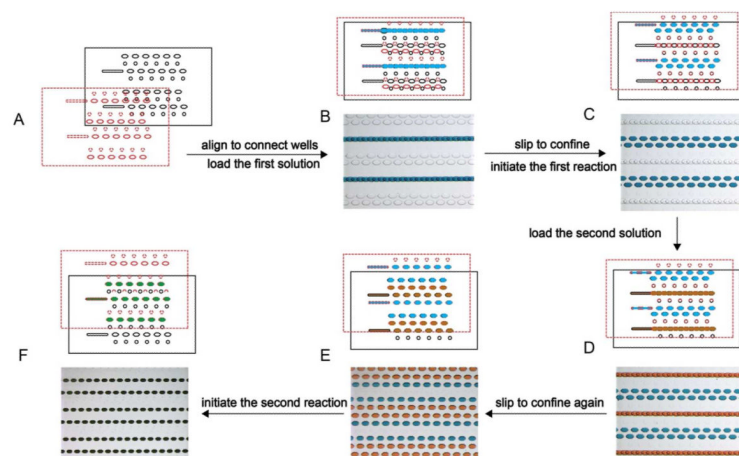
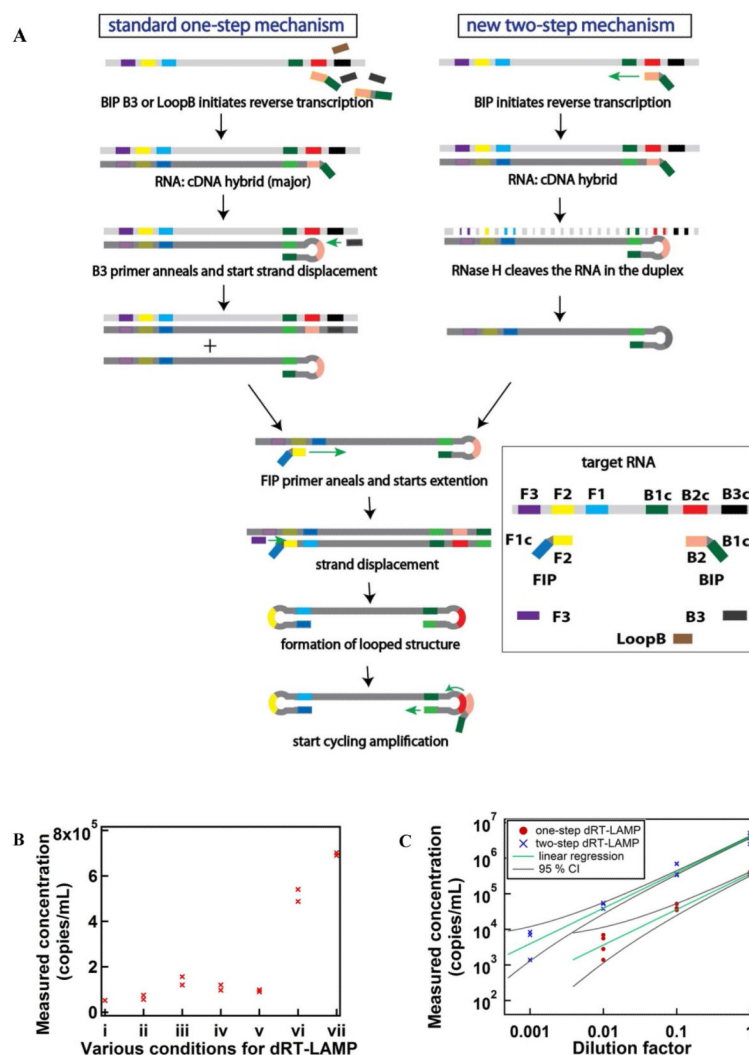


Figure 2.

Schematic drawings and images showing the operation of SlipChip for two-step dRT-LAMP. (A) Top (black) and bottom (red) plates of the SlipChip before assembly. (B) Assembled SlipChip after loading of RT solution. (C) RT solution containing RNA molecules confined to individual wells after slipping. (D) Loading of LAMP reagent mixture after RT reaction has completed. (E) LAMP reagent mixture confined to individual wells after slipping again. (F) Reaction initiated after slipping to mix RT and LAMP wells.

**Figure 3.**

A modified two-step mechanism for RT-LAMP leads to higher efficiency compared with a one-step protocol in digital format. (A) Comparison of the new two-step RT-LAMP mechanism versus the published one-step RT-LAMP mechanism. In the initial step, the two-step mechanism uses only BIP primer, the one-step protocol uses all primers together. After the formation of the DNA:RNA hybrid, the two-step mechanism uses RNase H instead of B3 primer initiated strand displacement to release the cDNA. The two mechanisms converge after the formation of first strand cDNA that is looped on one side. Figure truncated; details of cycling amplification including the loop primers' annealing are not shown here and can be found in previous papers.^{20,21} Inset shows designated primers—B2 and B2c, B3 and B3c, F1 and F1c are complementary sequences, respectively. (B) Concentration of HIV viral RNA (copies/mL) measured with dRT-LAMP using different protocols and the same template concentration. i) one-step dRT-LAMP; ii) two-step dRT-LAMP, all primers in RT step, AMV RT; iii) two-step, BIP in RT step, AMV RT; iv) two-step, BIP in RT step, Superscript III; v) two-step, BIP in RT step, AMV RT, with RNase H; vi) two-step, BIP in RT step, Superscript III, with RNase H; vii) two-step, BIP in RT step, Superscript III, with RNase H, 0.5× calcein (n=2). (C) Quantification results of HIV viral RNA (copies/mL) in a

serial dilution experiment on SlipChip using one-step dRT-LAMP and two-step dRT-LAMP (n=4). Error bars represent standard deviation.

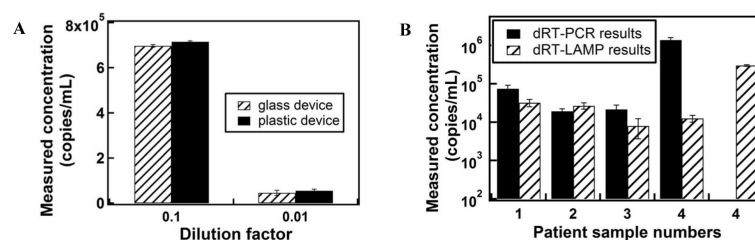


Figure 4.

(A) Quantification results of HIV viral RNA (copies/mL) on a plastic SlipChip at two dilution factors, with comparisons to results obtained on a glass device (n=3). (B) Quantification of HIV viral RNA purified from patient samples using dRT-LAMP and dRT-PCR. For sample #4, quantification results using dRT-LAMP with primers corrected for mutations are shown in the rightmost column of the figure (n=3). Error bars represent standard deviation.